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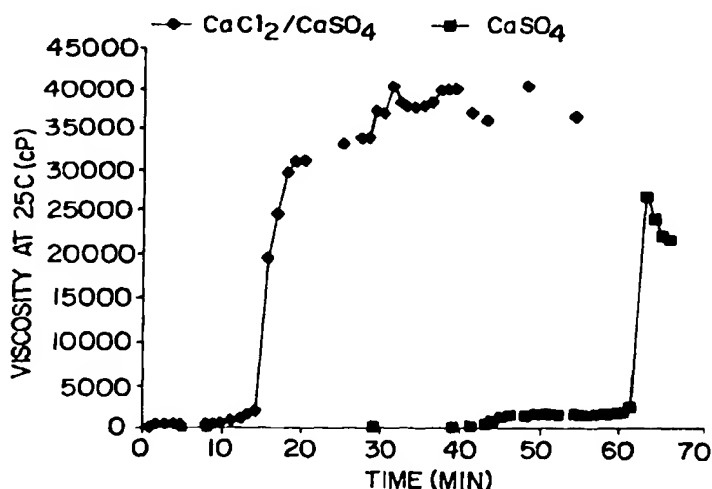
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(54) Title: IMPROVED HYDROGEL FOR TISSUE ENGINEERING



## (57) Abstract

This invention provides compositions for use in implanting into an animal comprising a biodegradable, biocompatible polymer which forms a hydrogel upon cross-linking. In a preferred mode, the polymer is cross-linked by multivalent ions; more preferably, a soluble salt of a multivalent ion and a sparingly soluble salt of a multivalent ion, these components being combined into a mixture which forms a partially hardened, injectable hydrogel, the consistency of the mixture being suitable for implanting the partially hardened hydrogel mixture into the animal, where the implanted, partially hardened hydrogel forms *in situ* a fully hardened hydrogel. Preferably, the mixture also contains a biocompatible sequestant which competes with the biocompatible polymer for binding of the multivalent cross-linking ion. The composition may or may not contain living cells. The invention also provides methods for implanting the composition in an animal, optionally as an implant containing living cells.

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-1-

## IMPROVED HYDROGEL FOR TISSUE ENGINEERING

This application is a continuation in-part of U.S. Ser. Nos. 08/762,733; 60/051,084; and 60/059,558, each of which is expressly  
5 incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### Field of the Invention

The present invention is directed to a process of implanting a  
10 suspension of cells and/or hydrogel particles for the creation of new tissue *in vivo*.

#### Review of Related Art

Contour deformities, whether traumatic, congenital, or aesthetic, currently require invasive surgical techniques for correction. Furthermore,  
15 deformities requiring augmentation often necessitate the use of alloplastic prostheses which suffer from problems of infection and extrusion. Collagen paste has been used to produce tissue bulk in certain application (e.g. 'Contigen' used to correct Intrinsic Sphincter Deficiency Incontinence), as has Teflon® paste (vesicoureteral reflux, vocal cord paralysis). However,  
20 collagen has been shown to degrade rapidly (6-12 months) *in vivo*, requiring multiple treatments to maintain bulk, and it is therefore unsatisfactory for correction of, e.g., craniofacial deformities. Similarly, alginate in other formulations has been shown to degrade in only months *in vivo*. Teflon paste particles have been demonstrated to migrate from the injection site,  
25 perhaps preferentially to the brain.

Techniques of tissue engineering employing biocompatible polymer scaffolds have been explored as a means of creating alternatives to prosthetic materials currently used in craniomaxillofacial surgery, as well as formation of organ equivalents to replace diseased, defective, or injured tissues. Tissue  
30 engineering involves the morphogenesis of new tissues from constructs formed of isolated cells and biocompatible polymers. Cells can be adhered

-2-

onto a polymeric matrix and implanted to form a cartilaginous structure. This can be accomplished, as described in U.S. Patent No. 5,041,138 to Vacanti, et al., by shaping of the matrix prior to implantation to form a desired anatomical structure and surgical implantation of the shaped matrix.

5       A simple method of delivering additional autogenous cartilage or bone to the craniofacial skeleton that is site specific and of controlled dimensions would reduce surgical trauma and eliminate the need for allografts or alloplastic prostheses. If one could transplant by means of injection or simple application to the desired site, and cause to engraft large  
10 numbers of isolated cells, one could augment the craniofacial osseocartilaginous skeleton with autogenous tissue but without extensive surgery. Furthermore, successfully implanting isolated cells would create the potential for tissue culture augmentation of the cells.

Transplantation via injection which can engraft large numbers of  
15 isolated cells, and augment the craniofacial osteo-cartilaginous skeleton with autogenous tissue, but without extensive surgery is clearly preferable. However, it has been shown that injection of dissociated cells subcutaneously or within areas of the body such as the peritoneum has not been successful. Cells are relatively quickly removed, presumably by  
20 phagocytosis and cell death.

Extending tissue engineering techniques to a system whereby the cell-polymer constructs are delivered less invasively and retained at the delivery site has been investigated to expand the applicability of tissue engineering to fields such as plastic surgery. Mixtures of dissociated cells and  
25 biocompatible polymers in the form of hydrogels have been used to form cellular tissues and cartilaginous structures which include non-cellular material that will degrade and be removed to leave tissue or cartilage that is histologically and chemically the same as naturally produced tissue or cartilage. Slowly polymerizing, biocompatible, biodegradable hydrogels  
30 have been demonstrated to be useful as a means of delivering large numbers

-3-

of isolated cells into a patient to create an organ equivalent or tissue such as cartilage. The gels appear to promote engraftment and provide three dimensional templates for new cell growth, and the resulting tissue can be similar in composition and histology to naturally occurring tissue.

5        Unlike the use of solid polymer systems to create a cell-polymer construct, a liquid composition that polymerizes to form a gel support matrix is more easily shaped and molded for custom reconstruction or augmentation. Additionally, a liquid polymer system can potentially be used for injectable delivery, which would be much less invasive than open  
10    implantation. Calcium alginate gels have been proposed as components in a means of delivering large numbers of isolated chondrocytes to promote engraftation and cartilage formation. These initial studies were extended in International Patent Publication No. WO 94/25080 to the formulating of slowly polymerizing calcium alginate gels and to the use of these gels to  
15    deliver large numbers of chondrocytes by means of injection, for the purpose of generating new cartilage.

      The endoscopic treatment of vesicoureteral reflux was first introduced in 1981 when polytetrafluoroethylene (Teflon®) was injected in the subureteral region of a patient. More than a decade later the search persists  
20    for an ideal injectable substance. Particle migration to distant organs raised concerns regarding the use of polytetrafluoroethylene paste. The high rate of re-treatment necessary due to implant volume loss has limited the usefulness of collagen. The ideal implant material should be nonmigratory, nonantigenic and delivered endoscopically, and should conserve its volume.  
25    Toward this goal, long-term studies to determine the effect of chondrocytes *in vitro* and *in vivo* determined that alginate, a biodegradable polymer, embedded with chondrocytes, would serve as a synthetic substrate for the injectable delivery and maintenance of cartilage architecture *in vivo*, and this system has been tested for the treatment of vesicoureteral reflux in a porcine  
30    model. However, the behavior of the system was less than satisfactory.

-4-

Injectable compositions containing cells have been produced by suspending suitable cells in medium M199, and mixing the cell suspension with an equal part of 2% sodium alginate solution, then adding solid calcium sulfate powder to initiate cross-linking of the alginate to form a hydrogel.

- 5 Such a composition typically will contain 1% sodium alginate in a hydrogel with insoluble calcium sulfate in an amount of about 200 mg per ml of suspension. A small amount of calcium chloride may be carried over into the composition from the cell suspension, but usually less than 0.1 mg per ml in the final suspension. Experience indicates that such suspensions have
- 10 a latent period on the order of an hour, followed by a rapid increase in viscosity to produce a relatively hard, even brittle gel within half an hour of the viscosity increase.

- However, the consistency of hydrogel-cell suspensions described above is not totally satisfactory for use in injection into patients in need
- 15 thereof. In some cases, the hydrogel-cell suspension hardens before it can be injected into the patient. Such gels are useful for preparing preformed structures for implantation into patients to repair structural defects of known shape, but require extensive surgery to open up a cavity of sufficient size to receive the preformed structure. On the other hand, some hydrogel-cell
- 20 suspensions maintain low viscosity until injection into the desired location in the body, but such suspensions assume the shape of the existing cavity or space in the patient. Such gels cannot be used where the defect is absence of a defined structure. Thus, there remains a need for a hydrogel-cell suspension with sufficient consistency to maintain its shape when implanted
- 25 in the patient, but which remains injectable to minimize the extent of surgery necessary for implantation. In particular, shortcomings in the present injectable cartilage formulation that would disallow its use in clinical trials include (a) insufficient viscosity to divide the tissue plane and not extravasate upon injection; (b) extended time required prior to achieving

-5-

high enough viscosity to attempt injection; and (c) inconsistent performance between lots due to poor distribution of components during formulation.

### SUMMARY OF THE INVENTION

5        It is an object of this invention to provide a composition which may be injected into a surgical site, but will assume and maintain a desired shape after injection. In particular, it is an object of this invention to provide an implantable composition having a consistency similar to POLYTEF<sup>TM</sup> teflon paste at injection which retains that consistency for a period of time  
10        sufficient to allow proper application.

      It is another object of this invention to provide an implant which will not migrate from the desired site, will retain its shape against gravity, and can be applied to a specific area by several means, including pumping through an injection cannula. These and other objects may be met by one or  
15        more of the following embodiments.

      In one aspect, this invention provides compositions for injection into an animal comprising a biocompatible polymer which forms a stable, partially hardened hydrogel. As used for injection into the animal, the composition is preferably a paste which, when formed into a block *in vitro*,  
20        resists flow under a force equal to the force of gravity. Typically the injectable paste is a suspension made up predominantly of irregularly shaped hydrogel particles, linear dimensions in the range of from 30 microns to 500 microns, where the injectable paste has gel strength of at least 0.1 kgf/cm<sup>2</sup> and the hydrogel in the particles has a gel strength of at least 3 kgf/cm<sup>2</sup>.  
25        Such a composition may be prepared by forming a friable hydrogel and forcing the hydrogel through an orifice whereby the hydrogel is fractured forming irregularly shaped particles; the resulting composition (containing the particles) will appear as a paste retaining consistency sufficient to separate tissue planes in an animal.

-6-

In a preferred mode, the biocompatible polymer in the composition of this invention is alginate, and the composition further comprises a highly soluble salt of a multivalent metal cation, a sparingly soluble salt of a multivalent cation. The highly soluble salt and the sparingly soluble salt  
5 may both be calcium salts. Alternatively, the highly soluble salt may be calcium chloride and the sparingly soluble salt may comprise a cation selected from the group consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin, and di-, tri- or tetra-functional organic cations and an anion selected from the group consisting of low molecular  
10 weight dicarboxylic acid ions, sulfate and carbonate. In a particularly preferred embodiment, the sparingly soluble salt is calcium sulfate and the mixture contains calcium chloride/calcium sulfate in ratio of from 0.001 to 100, more preferably from 0.0075 to 1.0, by weight. Preferably, the composition contains at least 0.5 % alginate by weight and at least about  
15 0.001 g calcium chloride per gram of alginate. In another preferred mode, the composition further comprises a sequestrant which competes with alginate for binding of calcium ion, and the sequestrant may comprise phosphate anion.

Optionally, the injectable composition of this invention which  
20 comprises a biocompatible polymer forming a hydrogel may also comprise living cells, such as chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells. Typically, the cells will be dissociated cells and/or cell aggregates.

25 In another aspect, this invention provides a kit for use in the method containing a biocompatible polymer capable of forming a hydrogel (or the polymer may be provided as an already-formed weakly cross-linked hydrogel) and an orifice through which the hydrogel may be forced, thereby fracturing the weakly cross-linked hydrogel (typically the orifice will be part  
30 of a syringe needle or cannula). In one embodiment, this invention provides



-7-

- a kit for preparing a partially hardened hydrogel composition suitable for injection into an animal which hydrogel will fully harden *in vivo*, where the kit contains (1) a biocompatible polymer, said biocompatible polymer being capable of forming a hydrogel upon cross-linking with multivalent cations,
- 5 (2) a highly soluble salt of a multivalent metal cation, and (3) a sparingly soluble salt of a multivalent cation. In a preferred mode, the kit contains (1) a polymer which forms a hydrogel upon cross-linking by divalent cations, a preferred polymer being alginate; (2) a fully soluble salt containing a divalent metal cation, a preferred soluble salt being calcium chloride; and (3)
- 10 a sparingly soluble salt having a cation selected from the group consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin, and di-, tri- or tetra-functional organic cations; and an anion selected from the group consisting of low molecular weight dicarboxylic acids, sulfate ions and carbonate ions, said sparingly soluble salt preferably being calcium sulfate.
- 15 The kit will provide the components such that upon combination of the components, optionally including a suspension of mammalian cells, the combined components will form within 30 minutes a partially hardened hydrogel suitable for injection, having the polymer at a concentration of 1.5%, the fully soluble salt at a concentration of 1.5 mg/L, and the sparingly
- 20 soluble salt at a concentration of 15 mg/L, and further wherein said partially hardened hydrogel will not under go substantial change in viscosity for at least 4 hours.

The components of the kit may be in separate containers, or two of more of the components may be combined in the kit. Optionally, the

25 biopolymer and at least one of the salts are in powder form, and in a preferred mode the biopolymer powder and the salt powder are combined in one container. More preferably the sparingly soluble salt and the biopolymer are both in powder form and are combined in one container. In another alternative, the kit further contains a sequestrant which competes

-8-

with alginate for binding of calcium ion, which may be in a separate container or may be combined with one or more other components.

In yet another aspect, this invention provides method for treating anatomical defects in an animal in need of treatment thereof, the method comprising implanting into the animal at the site of the defect a composition according to this invention. In one mode, the method for treating anatomical defects in an animal in need of treatment thereof comprises the steps of (1) preparing a biodegradable, biocompatible hydrogel which is partially hardened, the partially hardened hydrogel optionally containing living cells, (2) fracturing the hydrogel by passing the hydrogel through an orifice under pressure to produce a suspension containing hydrogel particles, and (3) implanting the suspension into the animal at the site of the defect, the hydrogel suspension after implantation inducing a fibrotic response by surrounding tissue and vascularization of the implant. Alternatively, the method for treating anatomical defects in an animal in need of treatment thereof comprises the steps of (1) preparing a biocompatible hydrogel comprising a biopolymer or a modified biopolymer, optionally containing living cells, (2) passing the hydrogel through an orifice under pressure to produce a flowable composition having consistency sufficient to divide tissue planes, and (3) implanting the flowable composition into the animal at the site of the defect.

In another embodiment, this invention provides a method for treating anatomical defects in an animal in need of treatment thereof comprising preparing a particulate suspension of biocompatible hydrogel which is partially hardened, and implanting the hydrogel suspension into the animal at the site of the defect, the hydrogel being provided as an intact, but weakly cross-linked gel (for example, gel strength =  $6.59 \text{ kgf/cm}^2$ ), which upon injection through a 22-gauge cannula at a rate of 10 ml/min. results in decreasing the gel strength (e.g., gel strength =  $0.231 \text{ kgf/cm}^2$ ). The composition used in this method forms a partially hardened hydrogel at room

-9-

temperature which is suitable for injection into an animal and which, after injection, will fully harden *in vivo*.

In a preferred embodiment, the present invention provides a readily available source of cross-linking cations, generally in the form of calcium chloride, at a level sufficient only to initiate and partially cross-link a biocompatible anionic polymer (e.g., alginate) to a thick paste consistency, preferably in approximately 15 minutes. More preferably, alginate is present in an amount sufficient to incorporate all water in the formulation into the loosely cross-linked gel (typically greater than about 0.5% alginate is present in such injectable cell suspensions). Such a formulation will set up quickly for prompt use in an operating theater, while maintaining good "injectable" consistency for an extended period of time to allow completion of procedures. Preferably, the partially cross-linked hydrogel will maintain this consistency in excess of 24 hours. Even more preferably, the consistency will be relatively stable for a sufficient period to allow the hydrogel to be prepared and marketed in partially cross-linked state. Generally, the suspension as injected will have a thick consistency which more easily divides tissues when injected and is less subject to extravasation from the injection site.

In a preferred embodiment, the invention provides a method for treating an anatomical defect in an animal by implanting, into the animal at the site of the defect, a suspension containing a biodegradable, biocompatible polymer which forms a hydrogel, typically upon cross-linking by multivalent ions, which are preferably in the form of a soluble salt of a multivalent ion, and a sparingly soluble salt of a multivalent ion, these components being combined so that the mixture forms a partially hardened, injectable hydrogel, where the partially hardened hydrogel forms an implant of the desired size and shape *in situ* and retains that size and shape for a period after implantation, and cells of the animal invade the hydrogel

-10-

implant to form a fibrous cell mass having substantially the same shape as the hardened hydrogel.

### **BRIEF DESCRIPTION OF THE FIGURES**

5           Figure 1 shows the time course of gel formation at 25°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium sulfate, respectively.

          Figure 2 shows the time course of gel formation at 37°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium  
10 sulfate, respectively.

          Figure 3 shows the time course of gel formation at 5°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium sulfate, respectively.

### **15 DETAILED DESCRIPTION OF THE INVENTION**

          This invention provides improved compositions that can be implanted into an animal in tissue engineering applications, as well as methods for implanting such compositions in an animal by injection. The composition of this invention comprises a biocompatible polymer in the form of a  
20 hydrogel. The compositions of this invention may be used without addition of living cells to the material to be implanted, in which case the implant will be invaded by cells of the surrounding tissue over time to produce a cellular mass having the shape of the original implant, or living cells may be included in the composition, in order to introduce other cell types to the  
25 region in which the composition is implanted by injection.

          Particular embodiments of this invention are directed to the production in animals of tissue-like bulk that persists long term, and the tissue-like material can mimic the characteristics of some soft tissues. The composition described for this invention can be injected to produce a  
30 persistent bulking material that will persist for a duration of sufficient length

-11-

to produce a practical correction of tissue voids resulting from injury, surgery, or inherited malformation. The composition can be applied through incision or injection and then shaped after application. The injected material will maintain shape, and subsequent fibroblastic proliferation and loose  
5 connective tissue invasion will produce a vascularized tissue to correct existing voids and to maintain the hydrogel composition where it is placed by injection. The applied gel material is invaded by loose connective tissue to maintain the volume and shape desired. This introduced material and resultant fibrotic influx persists for an extended period of time *in vivo*.

10 Gel according to this invention exhibits the property of inciting a fibrotic response. As described herein, injection of alginate gel into subcutaneous sites promotes an infiltration of loose connective tissue in and around the injected gel fragments. Through this response, connective tissue ultimately occupies a significant proportion of the bulk created upon  
15 injection of the gel material. Suitable compositions according to this invention are injectable pastes which typically contain irregularly shaped particles made up of a biocompatible polymer in the form of a hydrogel. The suspension will retain sufficient consistency (viscosity) to divide tissues when injected and to avoid extravasation from the injection site. It is  
20 believed that characteristics of the gel suspension which stimulate fibrotic response involve the particle size and that the hydrogel character of the particles is important for both stimulation of the fibrotic response and anchoring the implant in the injection site.

Preferably, the composition of this invention appears to be  
25 thixotropic. In other words, the hydrogel is partially hardened (weakly cross-linked), and as a result the hydrogel resists deformation up to a point; however, with increasing force, the composition will exhibit fluid behavior. In a preferred mode, a brittle hydrogel is formed and then fractured to form irregularly shaped particles of the desired dimension, for example by forcing  
30 the hydrogel through an orifice of suitable size. The resultant suspension of

-12-

hydrogel particles will hold its shape against the force of gravity. The suspension forms a paste that may be stacked upon itself to form a mound which will maintain substantially the same shape without other support. The resulting block or mound of paste will resist flow like a dab of toothpaste on a plate, although some rounding of the edges may be expected.

Preferably the hydrogel of this invention is a friable material which fractures when forced through an orifice such as a syringe needle or cannula. The consistency of the hardened hydrogel may change upon changes in the conditions. For example, increased calcium ion concentration will increase the degree of cross-linking for alginate, increased temperature will increase the cross-linking of PLURONICS™ hydrogel, etc. Once it has gelled, the partially hardened hydrogel according to this invention is stable, holding its shape against a force equal to or less than the effect of gravity for at least two hours, more preferably for at least twenty-four hours, and frequently for time periods measured in weeks. The fractured hydrogel will be a suspension of irregularly shaped particles whose size is a function of the orifice size and pressure. Typically, size will range from 30µm to 500µm, preferably from 40µm to 300µm. Suitable orifice size and injection pressures can be determined by the skilled artisan, and effective particle sizes can be confirmed by monitoring the fibrotic response as described in Example 6.

The hydrogel may be allowed to hardened inside the barrel of a syringe, and then exertion of pressure on the syringe plunger will force the composition to flow out of the syringe. In a typical formulation, the composition may be partially hardened and then forced through a 22 gauge cannula at a rate of 10 ml/min. as a high viscosity fluid without severe damage to any cells that may be included in the composition. In a suitable formulation, after flowing through the cannula the composition has the consistency of POLYTEF™ paste. For example, the composition may be a paste with a measured viscosity of at least 15,000 cPs at 25 °C , and when

-13-

the composition contains cells, injection of the composition through a 22-gauge cannula at a rate of 10 ml/min. will not substantially damage the cells (i.e., viability of the cell population reduced by more than 25 %).

Cell persistence and viability in the implant after injection and  
5 fibrotic reaction can be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes. The function of the cells can be determined using a combination of the above techniques and functional assays. Studies using labeled glucose as well as studies using protein assays can be performed to quantitate cell mass on the polymer  
10 scaffolds. These studies of cell mass can then be correlated with cell functional studies to determine what the appropriate cell mass is. In the case of chondrocytes, function is defined as synthesis of cartilage matrix components (e.g., proteoglycans, collagens) which can provide appropriate structural support for the surrounding attached tissues.

15 The biopolymer-containing composition of this invention is prepared and used in the form of a hydrogel or an aqueous suspension of hydrogel particles. Generally the hydrogel composition of this invention will contain an aqueous medium, which will usually include one or more pharmaceutically acceptable salts, and optionally other components. For  
20 example, when the biopolymer is alginate, the hydrogel may be formed by mixing a solution of alginate (e.g., soluble sodium alginate) with a solution containing multivalent ions in an amount sufficient to initiate cross-linking of the alginate to form a partially hardened hydrogel. The amounts and type of salts may vary, but usually the solutes and the solute concentration will  
25 be chosen to avoid undue stress on the animal when the composition is injected into the animal's tissues. When the hydrogel also contains living cells, the other components of the composition will be compatible with the living cells, preferably the composition will contain factors beneficial to growth and maintenance of the cells.

30

## Polymer

The polymeric material used in the implant according to this invention should form a hydrogel. A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) is cross-linked via  
5 covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure which entraps water molecules to form a gel. Examples of materials which can be used to form a hydrogel include polysaccharides such as alginate, carageenan, polyphosphazenes, and polyacrylates, which are cross-linked ionically, or block copolymers such as Pluronics™ or  
10 Tetronics™ polyethylene oxide-polypropylene glycol block copolymers which are crosslinked by temperature or pH, respectively.

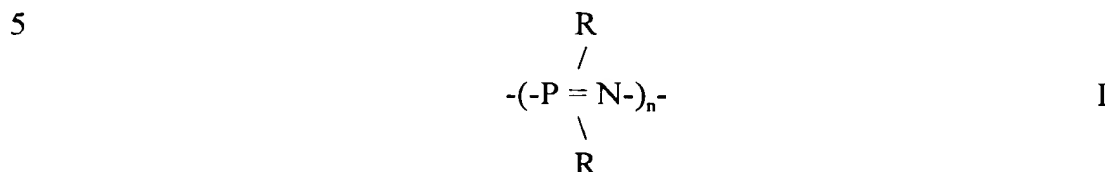
In general, these polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions, and the polymers may contain charged side groups, or a  
15 monovalent ionic salt thereof. Examples of polymers with acidic side groups that can be reacted with cations are poly(phosphazenes), poly(acrylic acids), poly(methacrylic acids), copolymers of acrylic acid and methacrylic acid, poly (vinyl acetate), and sulfonated polymers, such as sulfonated polystyrene. Copolymers having acidic side groups formed by reaction of  
20 acrylic or methacrylic acid and vinyl ether monomers or polymers can also be used. Examples of acidic groups are carboxylic acid groups, sulfonic acid groups, halogenated (preferably fluorinated) alcohol groups, phenolic OH groups, and acidic OH groups.

Examples of polymers with basic side groups that can be reacted with  
25 anions are polyvinyl amines), polyvinyl pyridine), polyvinyl imidazole), and some imino, substituted polyphosphazenes. The ammonium or quaternary salt of the polymers can also be formed from the backbone nitrogens or pendant imino groups. Examples of basic side groups are amino and imino groups.



-15-

Polyphosphazenes are polymers with backbones consisting of nitrogen and phosphorous separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two side chains ("R"). The repeat unit in polyphosphazenes has the general structure (I):



where n is an integer.

The polyphosphazenes suitable for cross-linking have a majority of side chain groups which are acidic and capable of forming salt bridges with dior trivalent cations. Examples of preferred acidic side groups are

15 carboxylic acid groups and sulfonic acid groups. Hydrolytically stable polyphosphazenes are formed of monomers having carboxylic acid side groups that are crosslinked by divalent or trivalent cations such as  $\text{Ca}^{2+}$  or  $\text{Al}^{3+}$ . Polymers can be synthesized that degrade by hydrolysis by incorporating monomers having imidazole, amino acid ester, or glycerol side

20 groups. For example, a polyanionic poly [bis(carboxylatophenoxy)] phosphazene (PCPP) can be synthesized, which is cross-linked with dissolved multivalent cations in aqueous media at room temperature or below to form hydrogel matrices.

Bioerodible polyphosphazines have at least two differing types of side

25 chains, acidic side groups capable of forming salt bridges with multivalent cations, and side groups that hydrolyze under in vivo conditions, e.g., imidazole groups, amino acid esters, glycerol and glucosyl. The term bioerodible or biodegradable, as used herein, means a polymer that dissolves or degrades within a period that is acceptable in the desired application

30 (usually *in vivo* therapy), less than about five years and most preferably less than about one year, once exposed to a physiological solution of pH 6-8 having a temperature of between about 25°C and 38°C. Hydrolysis of the

-16-

side chain results in erosion of the polymer. Examples of hydrolyzing side chains are unsubstituted and substituted imidazoles and amino acid esters in which the group is bonded to the phosphorous atom through an amino linkage (polyphosphazene polymers in which both R groups are attached in this manner are known as polyaminophosphazenes). For polyimidazolephosphazenes, some of the "R" groups on the polyphosphazene backbone are imidazole rings, attached to phosphorous in the backbone through a ring nitrogen atom. Other "R" groups can be organic residues that do not participate in hydrolysis, such as methyl phenoxy groups or other groups shown in the scientific paper of Allcock, et al., Macromolecules 10:824-830 (1977).

Methods for synthesis and the analysis of various types of polyphosphazenes are described by Allcock, H.R.; et al., Inorg. Chem. 11, 2584 (1972); Allcock, et al., Macromolecules 16, 715 (1983); Allcock, et al., Macromolecules 19, 1508 (1986); Allcock, et al., Biomaterials, 19, 500 (1988); Allcock, et al., Macromolecules 21, 1980 (1988); Allcock, et al., Inorg. Chem. 21(2), 515521 (1982); Allcock, et al., Macromolecules 22, 75 (1989); U.S. Patent Nos. 4,440,921, 4,495,174 and 4,880,622 to Allcock, et al.; U.S. Patent No. 4,946,938 to Magill, et al.; and Grolleman, et al., J. Controlled Release 3, 143 (1986), the teachings of which are specifically incorporated herein by reference.

Methods for the synthesis of the other polymers described above are known to those skilled in the art. See, for example Concise Encyclopedia of Polymer Science and Polymeric Amines and Ammonium Salts, E. Goethals, editor (Pergamen Press, Elmsford, NY 1980). Many polymers, such as poly(acrylic acid), are commercially available.

The water-soluble polymer with charged side groups is crosslinked by reacting the polymer with an aqueous solution containing multivalent ions of the opposite charge, either multivalent cations if the polymer has acidic side groups or multivalent anions if the polymer has basic side groups.

-17-

The preferred cations for cross-linking of the polymers with acidic side groups to form a hydrogel are divalent and trivalent cations such as copper, calcium, aluminum, magnesium, strontium, barium, and tin, although di-, tri- or tetra-functional organic cations such as alkylammonium salts, e.g.,

- 5  $R_3N^+ - \backslash \wedge \wedge / - ^+NR_3$  can also be used. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels and membranes. The higher the concentration of cation, or the higher the valence, the greater the degree of cross-linking of the polymer. Concentrations from as low as 0.005 M have been demonstrated to cross-link
- 10 the polymer. Higher concentrations are limited by the solubility of the salt.

The preferred anions for cross-linking of the polymers to form a hydrogel are divalent and trivalent anions such as low molecular weight dicarboxylic acids, for example, terephthalic acid, sulfate ions and carbonate ions. Aqueous solutions of the salts of these anions are added to the

15 polymers to form soft, highly swollen hydrogels and membranes, as described with respect to cations. The biocompatible polymer and the cross-linking agent may be dissolved in any physiologically compatible solvent(s). For examples herein, a common cell culture medium M-119 is use as a representative solvent.

- 20 A variety of polycations can be used to complex and thereby stabilize the polymer hydrogel into a semi-permeable surface membrane. Examples of materials that can be used include polymers having basic reactive groups such as amine or imine groups, having a preferred molecular weight between 3,000 and 100,000, such as polyethylenimine and polylysine. These are
- 25 commercially available. One polycation is poly (L-lysine); examples of synthetic polyamines are: polyethyleneimine, poly (vinylamine), and poly (allyl amine). There are also natural polycations such as the polysaccharide, chitosan.

Polyanions that can be used to form a semi-permeable membrane by

30 reaction with basic surface groups on the polymer hydrogel include polymers

-18-

and copolymers of acrylic acid, methacrylic acid, and other derivatives of acrylic acid, polymers with pendant  $\text{SO}_3\text{H}$  groups such as sulfonated polystyrene, and polystyrene with carboxylic acid groups.

Polymers that may be used in implants within the contemplation of this invention also include other natural, synthetic or modified biopolymers which under suitable conditions form hydrogels that are rheologically similar to the hydrogels described herein. Selection of suitable biopolymers is within the skill of the ordinary artisan, in view of the hydrogel characteristics described herein, and suitability may be confirmed based on performance of the polymer in assays and tests described herein. Such polymers must be biocompatible and may be non-biodegradable or biodegradable over a period of days, weeks or even years. Suitable biopolymers include, for example, modified alginates and other modified biopolymers (see, e.g., Putnam A J; Mooney D J (1996), "Tissue engineering using synthetic extracellular matrices," *Nature Medicine*, 2(7):824-826, (1996); Mooney, D.J. (1996), "Tissue engineering with biodegradable polymer matrixes," in Bajpai, Praphulla K (Ed), Proc. South. Biomed. Eng. Conf., 15th, IEEE, NY, 1996; and Wong, W.H., and D.J. Mooney, "Synthesis and properties of biodegradable polymers used as synthetic matrices for tissue engineering, in Atala, et al., eds., *Synthetic Biodegradable Polymer Scaffolds* (Tissue Engineering), Birkhauser, 1997, ISBN:0817639195).

#### **Alginate Biopolymer Compositions**

In a preferred embodiment, the method of this invention involves formation of hydrogel by multivalent cation cross-linking of acidic biopolymers in which the availability of the multivalent cation is controlled by dissolution of a sparingly soluble salt to replenish soluble cation as the soluble cation is absorbed in cross-links. A portion of the multivalent cation is provided by a fully soluble salt and is freely available ("fast" cross-linking ion), while another portion of the multivalent cation necessary for full cross-linking is provided in a sparingly soluble salt ("slow" cross-linking ion).

-19-

The preferred polymer is alginate for this embodiment; however, the method of this invention could also apply to various acidic polymers, such as the biopolymer  $\kappa$ -carrageenan. In the following discussion, alginate should be considered representative of any suitable polymer.

5           For use with the preferred biopolymer (alginate), the preferred multivalent ion, is calcium. The method of this invention is described herein using calcium and calcium salts as representative cross-linkers for controlling gelation characteristics and final gel properties using multiple cation sources to supply cross-linking cations, where the cation sources are  
10 chosen for disparate solubilities. Other multi-valent ions which perform similarly are, of course, within the contemplation of this invention. The composition provided by this invention preferably also contains a soluble buffering system for the multivalent cation, usually phosphate when the cation is a di- or tri-valent metal ion. Phosphate competes with alginate for  
15 the metal, so that cross-linking of the alginate in the composition is forestalled by competition between alginate and phosphate anions for the limited amount of available cation as the components of the composition are mixed.

          In an exemplary embodiment, the biocompatible acidic biopolymer  
20 alginate is cross-linked by a minor portion of fully-soluble calcium chloride and a major amount of the much-less-soluble calcium sulfate. The composition from which the hydrogel is formed contains phosphate anion in relatively high concentration to buffer the soluble calcium ion concentration and forestall complete cross-linking of the alginate. Once the composition  
25 is injected at the desired site, phosphate anion is diluted out by diffusion into the surrounding biological fluids, and the concentration of soluble calcium ion increases locally. As the soluble calcium ion concentration increases, more calcium cross-links are formed in the alginate, and the hydrogel hardens. Similar effects may be obtained for analogous metal ions by  
30 buffering with phosphate anion or other similar systems, e.g., biologically

-20-

compatible sequestrants, which buffer soluble cation concentration in an analogous manner.

Alginate is a copolymer of stretches of polymannuronate and polyguluronate obtained from seaweed. Suitable alginate is available from commercial sources. Individual alginate preparations will have a determinable capacity for binding calcium which is a function of the mannuronate:guluronate (M/G) ratio. Effects resulting from varying the ratio between mannuronate and guluronate residues are described in U.S. Patent No. 4,950,600, incorporated herein by reference. A suitable, highly purified alginate has M/G ration of 35/65.

Alginate can be ionically cross-linked with divalent cations, in water, at room temperature, to form a hydrogel matrix. Due to these mild conditions, alginate has been the most commonly used polymer for hybridoma cell encapsulation, as described, for example, in U.S. Patent No. 4,352,883 to Lim. In the Lim process, an aqueous solution containing the biological materials to be encapsulated is suspended in a solution of a water soluble polymer, the suspension is formed into droplets which are configured into discrete microcapsules by contact with multivalent cations, then the surface of the microcapsules is crosslinked with polyamino acids to form a semipermeable membrane around the encapsulated materials.

Cell/polymer systems in hydrogel form are used for tissue engineering, including suspensions of cells such as chondrocytes in alginate solution to which calcium salts are added to initiate hydrogel formation. Typical of these systems are chondrocyte/calcium alginate solutions which are created by first vortexing an isolated cell suspension with sodium alginate solution (e.g., in 0.1M  $K_2HPO_4$ , 0.135 M NaCl, pH 7.4) to yield a cellular density of  $20 \times 10^6$  cells/mL (a cellular density of approximately 50 percent of that of native articular bovine cartilage) in a 1.0% alginate solution. The chondrocyte-sodium alginate suspension may be stored on ice

-21-

at 4°C until use, and prior to injection, 0.2 gm of sterilized CaSO<sub>4</sub> powder is added to each milliliter of the cold chondrocyte-alginate solution.

Studies on the gel formation properties of formulations using calcium sulfate as the source of cross-linking ion (see, e.g., Example 1) show that such formulations can gel in as little as 16 minutes at human body temperature, but do not gel readily at room temperature. When the temperature of the alginate/cell suspension is raised to a temperature at which gel formation is initiated within a reasonable time (i.e., on the order of minutes rather than hours in the operating room), a window of only several minutes exists during which the consistency of the composition is suitable for injection. Failure of the formulation to gel at room temperature necessitates the use of heating equipment for incubation of the formulation prior to injection. Additionally, mixing and injecting problems occur due to the formation of gel around CaSO<sub>4</sub> particles which blocks the transfer of Ca<sup>+2</sup> through the solution, and further blocks injection through a syringe cannula by forming clots of alginate-encapsulated CaSO<sub>4</sub>. Therefore, a filter must be attached to the syringe in order to screen out lumps created by more rapidly gelling regions in the suspension. These characteristics increase the complexity in the formula's clinical application and make it difficult to achieve any lot-to-lot consistency in delivery and gelling properties.

To avoid the requirement for heating the mixture plus adding a filter on the injection syringe, the present inventors have developed a new formula for injectable hydrogels used in tissue engineering. This formula provides for accelerated gel formation and prolonged injectability of the gel suspension, leading to more consistent performance.

To rapidly generate sufficient consistency to divide tissue planes upon injection and to prevent extravasation while avoiding the problems of blocked injection cannulas, this invention provides a hydrogel forming composition containing two sources of cross-linking polyvalent cation: a minor amount of one cation source which provides fully available cation and

-22-

a major amount of another cation source which provides slow release of the cross-linking ion as the gel is formed. The first cation source is a highly water soluble salt - i.e., solubility of at least 1g per 100 ml, preferably at least 30g/100ml, more preferably at least 60g/100ml. Particularly preferred is a calcium salt such as calcium chloride. The second cation source is a sparingly soluble salt - i.e., solubility less than 1g/100ml, preferably less than 0.5g/100ml, more preferably less than 0.3g/100ml. Particularly preferred is a calcium salt such as calcium sulfate. The solubilities of calcium sulfate dihydrate ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) in cold and hot ( $100^\circ\text{C}$ ) water are 2.41 and 2.22 mg/mL, respectively. Any two salts of multivalent cations having similar water solubilities to calcium chloride and calcium sulfate, respectively, are also suitable for use in this invention. While either salt alone may provide sufficient cross-linking cation to produce a fully hardened alginate hydrogel, use of two salts spreads the cross-linking action over time, thereby expanding the time window during which a partially hardened hydrogel (weakly cross-linked hydrogel) is of suitable consistency for injection through a cannula.

Calcium chloride is one preferred "fast"  $\text{Ca}^{+2}$  source to speed up the alginate gelling because of its high solubility. Other calcium salts of equivalent solubility work in a similar fashion. Experiments were run to determine if  $\text{CaCl}_2$  alone could work. A concentration range of 1.0 to 6.6 mg/mL was tried, and it was found that  $\text{CaCl}_2$  alone was not suitable to cross-link the hydrogel, since the alginate gelled up immediately at the interface of alginate and  $\text{CaCl}_2$  solutions when the concentration of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  is equal to or higher than 3.3 mg/mL, but would not gel up at all when equal to or lower than 2.7 mg/mL when alginate was used at a final concentration of 1.5% (w/v). Depending on alginate concentration and desired gel strength,  $\text{CaCl}_2$  may range from 0.5mg/mL to 3.0 mg/mL.

The amount of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  required to react stoichiometrically with a typical sodium alginate is 0.309 mg per mg of alginate. A typical known calcium sulfate cross-linked formulation uses 20 mg  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  powder



-23-

per mg of sodium alginate, or approximately 65 times the amount required. Mixing and injecting problems occur however, due to the formation of gel around  $\text{CaSO}_4$  particles which blocks the transfer of  $\text{Ca}^{+2}$  through the solution and further blocks injection through a syringe cannula by forming

5 clots of alginate-encapsulated  $\text{CaSO}_4$ .

Proportions are preferred which produce a homogeneous gel of acceptable consistency in required time. The preferred ratio of  $\text{CaSO}_4/\text{CaCl}_2$  depends on the polymer (e.g., the M/G ratio for alginate), the required time for gelation, required texture strength, etc. As an example, for

10 a sodium alginate which can bind 0.309 g of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  per g of alginate, the ratio of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{CaSO}_4 \cdot 2\text{H}_2\text{O}/\text{alginate}$  can be 1:10:10, preferably with certain amounts of phosphate present in the formulation.

Suitable compositions may have proportions which produce a gel of acceptable consistency in approximately 14 minutes at room temperature.

15 The ratio of  $\text{CaSO}_4/\text{CaCl}_2$  which provides about 3 mg calcium chloride and 30 mg of calcium sulfate per 30 mg of alginate is suitable for use with alginate which binds 0.309 g of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  per g of sodium alginate. The stoichiometry for alginate with a different calcium binding capacity can be readily determined by one skilled in the art, and similar mole ratios of

20 sparingly soluble and freely soluble salts can be calculated to determine suitable amounts of these alternative salts. M/G ratio of the alginate determines the Ca-binding capacity. Preferably, the relative amounts of alginate, fully soluble salt and sparingly soluble salt, and optionally soluble sequestrant, will be such that the mixture forms a gel within 20 minutes that

25 will hold its shape against the force of gravity, but may be injected without clogging the injection cannula for at least 4 hours. (These times are for incubation of the mixture at room temperature.)

As discussed above, formulations using only slowly soluble cation sources produce a partitioning of components into alginate-encapsulated

30 clods of undissolved salt and a more liquid portion that could be collected and injected. Such a product is poorly reproducible in consistency, cannot

-24-

be well-characterized, well-controlled, or altered to give the final formulation a range of properties. Use of only rapidly soluble cation sources can produce partitioning as well, if the cation cannot be distributed throughout the mixture, as portions of fully cross-linked gel will be produced and are not easily injected. Addition of both a sparingly soluble and a freely available multivalent cation source may circumvent these problems. However, the inventors have found that mixing the alginate with two cation sources in the presence of a cation-sequestering anion which provides an excess of cation binding capacity produces a final formulation that also demonstrates an improvement over existing gels: notably homogeneous mixing and production of a consistent suspension, with controlled and uniform properties throughout, that is completely injectable.

Consequently, the hydrogel forming composition according to this invention optionally contains a component which will buffer the amount of cross-linking cation available to the biopolymer. The buffering component is usually an anion sequestrant such as phosphate anion. Rapid and complete cross-linking of the alginate is prevented by the  $\text{PO}_4$  anions in the formula competing with alginate in binding of  $\text{Ca}^{+2}$ , which greatly reduces the gelation speed. Phosphate anion is typically present at about 0.1M, but the phosphate concentration may be varied from 0.03M to 0.3M to control gelling time. Other biologically compatible sequestrants which will complete with alginate for calcium, and thus prevent formation of alginate encapsulated salt particles in a similar manner, are also contemplated by this invention.

An excess of cation binding capability, and competition between anions and alginate for that available cation, permits a complete distribution of the available cation throughout the suspension. The product is homogenous throughout, is produced consistently, can be characterized by conventional testing protocols, is entirely available for application without product loss, and can be adjusted to impart desired properties (e.g. viscosity, gel strength, gelation time, etc). This uniformity in mixing is demonstrated

-25-

in different mixing formats, such as vortexing or hand mixing in test tubes, magnetic and mechanical stirrers, within mixing syringes, etc.

The rate of degradation of alginate is not well documented, and depends, e.g., on the method of cross-linking, type of alginate, and site of administration. Certain formulations of alginate gels are only slowly broken down in the human body due to lack of enzymes that recognize the polysaccharide. Furthermore, alginate can be modified and/or cross-linked with various agents to increase or decrease its rate of degradation. Alginate can be cross-linked using two calcium sources so as to produce a weak gel that can be injected or applied to tissue spaces or voids and be shaped or molded to conform to a desired shape. Production of suitable compositions (except for the addition of cells may be omitted in formulations of the present invention) is described in detail in U.S. Serial No. 08/762,733, which is incorporated herein by reference in its entirety.

Coupling the characteristics of alginate degradation and the fibrotic response to the formulation described herein, the present invention produces an enduring tissue-like bulk that maintains its injected dimensions, does not migrate, and which may persist for a period of time significantly greater than current tissue bulking agents. A particularly beneficial characteristic is that the material can be injected through needles of many dimensions, allowing cystoscopic or endoscopic application, with little surgical intervention.

#### Sources of Cells.

The compositions of this invention can be used to provide multiple cell types, including genetically altered cells, within a three-dimensional scaffolding for the efficient transfer of large number of cells and the promotion of transplant engraftment for the purpose of creating a new tissue or tissue equivalent. It can also be used for immunoprotection of cell transplants while a new tissue or tissue equivalent is growing by excluding the host immune system.

-26-

Examples of cells which can be implanted as described herein include chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells, and also includes expanded populations of isolated stem cells. As used herein, "organ cells" includes hepatocytes, islet cells, cells of intestinal origin, cells derived from the kidney, and other cells acting primarily to synthesize and secrete, or to metabolize materials.

The cells to be encased in the injectable hydrogel can be obtained directly from a donor, from cell culture of cells from a donor, or from established cell culture lines. In the preferred embodiments, cells are obtained directly from a donor, washed and implanted directly in combination with the polymeric material. Cellular material may be dissociated single cells, minced tissue (i.e., clumps of aggregated cells) or cell aggregates generated *in vitro* from dissociated cells. The cells may be cultured using techniques known to those skilled in the art of tissue culture.

Cell persistence and viability after injection can be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes. The function of the implanted cells can be determined using a combination of the above-techniques and functional assays. For example, in the case of hepatocytes, *in vivo* liver function studies can be performed by placing a cannula into the recipient's common bile duct. Bile can then be collected in increments. Bile pigments can be analyzed by high pressure liquid chromatography looking for underivatized tetrapyrroles or by thin layer chromatography after being converted to azodipyrroles by reaction with diazotized azodipyrroles ethylanthranilate either with or without treatment with P-glucuronidase. Diconjugated and monoconjugated bilirubin can also be determined by thin layer chromatography after alkalinemethanolysis of conjugated bile pigments. In general, as the number of functioning transplanted hepatocytes increases, the levels of conjugated bilirubin will increase. Simple liver function tests can also be done on blood samples, such as albumin production.

-27-

Analogous organ function studies can be conducted using techniques known to those skilled in the art, as required to determine the extent of cell function after implantation. For example, islet cells of the pancreas may be delivered in a similar fashion to that specifically used to implant  
5 hepatocytes, to achieve glucose regulation by appropriate secretion of insulin to cure diabetes. Other endocrine tissues can also be implanted. Studies using labeled glucose as well as studies using protein assays can be performed to quantitate cell mass on the polymer scaffolds. These studies of cell mass can then be correlated with cell functional studies to determine  
10 what the appropriate cell mass is. In the case of chondrocytes, function is defined as synthesis of cartilage matrix components (e.g., proteoglycans, collagens) which can provide appropriate structural support for the surrounding attached tissues.

Precursor cells of chondrocytes, or cells derived from pluripotent  
15 stem cells which have the capability of differentiating into chondrocytes, can also be used in place of the chondrocytes. Examples are fibroblasts or mesenchymal stem cells which differentiate to form chondrocytes. As described herein, the term "chondrocytes" includes such chondrocyte precursor cells.

20

### Mixing protocols

Substantial variation is permitted in the order of addition for the components which react to form the hydrogel having partially hardened consistency. Typically alginate, optionally including the sequestering anion,  
25 is mixed with the cation sources in one or more steps. Generally, a biodegradable liquid polymer is used, such as alginate which is designed to solidify at a controlled rate upon subsequent mixing with multivalent ionic salts. Variations in the levels of individual components, or conditions of mixing and incubation can be modified to a) control the consistency of the  
30 material as injected; b) control the time required for attaining an injectable consistency; c) and controlling the properties of the final gel *in vivo* to

-28-

accommodate particular requirements of the receiving tissue site for appropriate texture and dimensional retention.

Levels of individual components, either singly or in combination, can be modified to alter different properties of the formulation both before and after application so as to accommodate particular requirements a) for injection and application, b) for successful engraftment of the implant and creation of required properties and function of the final gel and replacement tissue, or c) for the manufacture, distribution, and application to patients of the formulation. For example, injection of a hydrogel formulation into compact tissues (e.g. muscle, submucosa) requires a high viscosity to prevent extravasation of material. Alterations of viscosity can be achieved by a number of mechanism either singly or in concert, such as (1) selection of the viscosity of the raw material (e.g. low, medium, or high viscosity alginate); (2) concentration of gel (e.g. a range of 0.3% to 3.0% alginate can be used to achieve a broad range of gel viscosities); (3) amount of highly soluble multivalent cation source to control degree of partial cross-linking; or (4) level of anions provided to compete with alginate for cation binding. Successful invasion of the implant by different cell types, or the nature of the desired replacement tissue to be created may require alterations of the formulations components. For example, creation of a firm, compact tissue (e.g., liver, cartilage) requires a gel of higher polymer chain length or concentration (e.g., >1.5% alginate and/or high guluronic content alginate).

Controlling the duration of injectability and properties of a formulation prior to injection would aid the manufacture, distribution, and application of therapies according to this invention. The working time of the final formulation, or the time required for materials to setup to an injectable consistency, can be controlled through multiple methods, such as by the amount of highly soluble cation provided and/or the level of anion provided to compete for cation binding, in addition to temperature regulation. For example duration of an injectable consistency can be controlled through temperature so as to allow a working time of minutes (34-38°C) or beyond

-29-

one month (4-8°C). Temperature plays a very important role in the gel formation speed. Upon increasing the temperature, as will occur, for instance, when the material is injected into tissue having a temperature of 37°C, the material will proceed to harden into a fully cross-linked hydrogel.

- 5 As shown in Example 1, at  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  level of 0.1 x (i.e. 20 mg/mL in a formulated batch) the partially hardened gel could set up in 32 minutes at body temperature and remain injectable for at least 90 minutes. This best case scenario of  $\text{CaSO}_4$  at 37 °C still requires excessive time to reach injectable consistency, and is both more complicated and more inconsistent  
10 than desired.

- Cation sources can be chosen to control gelling and gel properties, and also combined to control degradation. The composition according to this invention, having both a fast and slow source of cross-linking agent, can provide both more rapid viscosity increase, and a longer time before  
15 becoming fully cross-linked compared to the formulation with calcium sulfate alone. In a particularly preferred mode, the mixture which forms partially hardened hydrogel will also include a significant amount of phosphate anion. Typically, the phosphate concentration will be approximately 0.1 molar.

- 20 Increasing the concentration of alginate is also helpful for the gel to hold more water, resulting in a thicker paste and a stronger gel, and also in accelerating the gel formation. Preferred alginate level is at least 0.75%, more preferably between 1.5% and 2.5% and up to 3.0%, in the final cell-containing suspension which forms the hydrogel.

- 25 In a preferred embodiment of this invention, the acidic biopolymer is sodium alginate, the fully soluble salt is calcium chloride, and the sparingly soluble salt is calcium sulfate. The alginate is present in the amount of at least 0.75% by weight of solution, preferably about 1.5%. The freely soluble salt provides 10 to 15 mM calcium ion, while the sparingly soluble  
30 salt is provided as a powder dispersed in the alginate solution in an amount which would supply approximately 8 times more calcium ion if it were

-30-

completely dissolved. In other words, calcium is supplied as the divalent metal cross-linking agent by two salts, calcium chloride and calcium sulfate, in a ratio of 1 part to 10 parts by weight. In a preferred method of preparing the hydrogel suspension, 9 parts of a 2% alginate solution are mixed with 2  
5 parts of a suitable cell suspension medium such as M-199, and subsequently 1 part of the same cell suspension medium containing 1.8% anhydrous calcium chloride with 18% calcium sulfate suspended therein is added to the alginate-calcium chloride mixture. The final mixture is mixed thoroughly and used for injection into tissue. A rapid increase in viscosity may be  
10 expected within 15 minutes of mixing all of the components together. The consistency of this mixture should be sufficient for the mixture to hold its shape against gravity, but will remain injectable at room temperature for at least 24 hours.

A preferred viscosity for injectable application varies by intended use,  
15 but is a balance of parameters that include (a) the formulation reservoir (e.g., syringe size, piston diameter, resistance required for proper "touch" or application rate), (b) application device (catheter or needle length, diameter, composition), (c) receiving tissue resistance to division, and (d) type of distribution required (e.g., topical application to bone or organ surface,  
20 injection within tissues without extravasation, etc.).

Also within the contemplation of this invention are kits for use in preparation and injection of the compositions taught herein. Such kits include the components of the composition in one or more containers and may contain a formulation reservoir (e.g., mixing vessel or syringe) and/or  
25 a gel fracture orifice (e.g., cannula or syringe needle).

#### **Therapeutic Application.**

Typically, a hydrogel solution is injected directly into a site in a patient, where the hydrogel forms an implant and induces fibrotic response  
30 from the surrounding tissues. The loose connective tissue formed by the fibrotic response holds the hydrogel particles in place, and tissue formation



-31-

is accompanied by vascularization, producing vascularized mass of soft tissue consistency occupying the volume established during implantation. Such hydrogel implants can be used for a variety of reconstructive procedures, including molding of implants *in situ* to reconstruct three dimensional tissue defects.

Alternatively, cells may be included in the hydrogel composition of the present invention and injected directly into a site in a patient, where the hydrogel hardens into a matrix having cells dispersed therein. In another alternative, cells may be suspended in a biopolymer solution which is poured or injected into a rigid or inflatable mold having a desired anatomical shape, then hardened to form a matrix having cells dispersed therein which can be implanted into a patient. After implantation, the hydrogel ultimately degrades, leaving only the resulting tissue. Such hydrogel-cell mixtures can be used for a variety of reconstructive procedures, including custom molding of cell implants to reconstruct three dimensional tissue defects, filling pre-inserted inflatable molds or scaffolds, as well as implantation of tissues generally. The present invention contemplates such implants using the composition according to this invention.

Treatment of vesicoureteral reflux and incontinence has been described where chondrocytes, preferably autologous chondrocytes, are mixed with a liquid biodegradable biocompatible polymeric material, such as alginate which can be solidified *in vivo*, or other carrier to form a cell suspension. The hydrogel suspension of this invention may be injected into the area where reflux is occurring, or where a bulking agent is required, in an amount effective to induce formation of tissue bulk that provides the required control over the passage of urine. The suspension contains a biodegradable liquid polymer such as alginate, a copolymer of guluronic and mannuronic acid, which is designed to solidify at a controlled rate when contacted with calcium salts. The hydrogel composition is injected at the desired site where it forms a tissue-like bulk to correct the defect. The hydrogel composition according to this invention may also contain cells such

-32-

as chondrocytes to supplement the cellular invasion from the surrounding tissue. The cell suspension contains chondrocytes, harvested, grown to confluence, passaged as needed, and then mixed with a biodegradable liquid polymer such as alginate, a copolymer of guluronic and mannuronic acid, which is designed to solidify at a controlled rate when contacted with calcium salts. The cells are mixed with hydrogel forming compositions and then injected at the desired site where they proliferate and correct the defect. Autologous chondrocyte cells are preferred, because with autologous cells, this method of treatment does not require FDA approval.

Hydrogel based on weakly cross-linked polymeric material, such as alginate, can be injected into the area of the defect in an amount effective to yield an elevation of the bladder mucosa that corrects the defect, for example, which provides the required control over the passage of urine. Again, a biodegradable polymer serves as a synthetic substrate for the injectable implant for maintenance of tissue architecture in humans. The alginate suspension can be easily injected cystoscopically, and the tissue formed is able to correct vesicoureteral reflux without any evidence of obstruction.

The ideal injectable substance for the endoscopic treatment of reflux should be a natural bulking agent which is non-antigenic, non-migratory, and volume stable. The procedure can be performed under 15 minutes, with a short period of a mask anesthetic, in the outpatient unit, without any need for a hospital stay. Neither vesical nor perivesical drainage is required. Since the whole procedure is done endoscopically and the bladder is not entered surgically, there is no postoperative discomfort whatsoever. The patient can return to a normal level of activity almost immediately.

The gel material described herein can be formulated from sterile components at a manufacturing site and supplied to a physician either as separate units, which may be combined to form the final gel material, or as a pre-loaded syringe containing the final cross-linked product.

-33-

This material may be used to replace existing tissue bulking agents; e.g. collagen pastes; Teflon pastes, in applications such as vocal cord paralysis and soft tissue injury voids.

5

### EXAMPLES

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

#### 10 **EXAMPLE 1. Effect of Calcium Sulfate Concentration on Gel Time**

This experiment shows the effect of changing calcium sulfate concentrations in the absence of calcium chloride. Calcium sulfate levels were varied from 0.02 g/mL (0.1x) through 0.2 g/mL (1.0x) at 0 °C, 37 °C, and room temperature (23 °C), while keeping other content of the formula  
15 unchanged. Sodium alginate, grade UP MVG from Pronova with M/G ratio of 35/65, was prepared as 2% solution in 0.1 molar phosphate buffer, pH=7.4, containing 0.79% sodium chloride. Sodium alginate solution was diluted 1 to 1 with M199 cell culture medium, and calcium sulfate powder was added in amounts ranging from 20 mg/mL to 200 mg/mL. After mixing,  
20 sample solutions were held at either ice-bath temperatures (0°C), room temperatures (R.T.), or body temperature (37°C). Each sample was tested periodically for gel formation by injecting an aliquot onto a mylar sheet.

The results are shown in Table A. The time for gel formation (indicated as X) and for blocking injection (marked B) is also shown. As  
25 can be seen from Table A, in the absence of added calcium chloride, no gel formation was observed on the ice-bath. At room temperature, gel formation occurred between 70 and 100 minutes, and the hydrogel injection was blocked within 20 minutes of that time period. At 37°C, the initial gelation occurred from 32 minutes down to 16 minutes depending on the  
30 concentration of calcium sulfate; blockage of injection generally occurred within 15 minutes of initial gelation.

-34-

The experiments show that at 0 °C, no gelling (nc) occurred after 120 minutes. At room temperature, gelation started at 90 min for 0.2x and 70 min for 1.0x formula. For body temperature, gelation started at 16 min for 1.0x formula, and the gel setting time decreases almost linearly with the decrease in calcium sulfate concentration. Although the calcium sulfate cross-linked hydrogel formula can gel up in 16 minutes at human body temperature, it allows only several minutes for injection, plus it necessitates the addition of heating equipment and a filter attached to the syringe in order to achieve any lot-to-lot consistency in delivery and gelling properties. Both of these requirements will increase the complexity in the formula's clinical application. Temperature plays a very important role in the gel formation speed. From Table A, at  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  level of 0.1 x, i.e. 60 mg in a formulated batch, the gel could set up in 32 minute at body temperature and remain injectable for at least 90 minutes. This best case scenario of  $\text{CaSO}_4$  at 37 °C still requires too much time until injection, and is both more complicated and more inconsistent than desired.

Table A. Alginate Gel Set-up and Injectability Experiment

Temp	CaSO <sub>4</sub>	Min	4	8	12	16	18	20	22	24	28	32	36	40	50	60	70	80	90	100	120
C	x0.2g/ ml																				nc
	0.1x																				nc
	0.2x																				nc
0	0.4x																				nc
	0.6x																				nc
	0.8x																				nc
	1.0x																				nc
	0.1x																				nc
	0.2x																	x			
R.T.	0.4x																	x	B		
	0.6x																			x.B	
	0.8x																	x.B			
	1.0x																x		B		

[illegible]

Note: x = cross-link, B = block, nc = no cross-link, R.T. = room temperature. Blocked needles might have been caused by  $\text{CaSO}_4$  particles and resulting gel formed around these particles. Each sample contains 1.5 ml 2% Sodium Alginate in pH 7.4 buffer, 1.5 ml M199 Medium (no cells), and indicated amounts of  $\text{CaSO}_4$  powder. Materials are stored on ice prior to mixing. After mixing, samples are stored at the indicated temperature, and injected at the indicated time onto mylar sheet.

**Example 2. Mixtures of Calcium Chloride and Calcium Sulfate.**

In a preferred formulation, sodium alginate is dissolved in pH 7.4  $K_2HPO_4$  buffer. Cells are supplied in M199 medium and then suspended in the alginate solution. Calcium sulfate powder is stored in a 1.5 ml vial.

- 5 Calcium chloride is dissolved in M199 medium with concentration of 18 mg/mL and stored in another vial. In the application, the  $CaCl_2$  solution will be mixed with  $CaSO_4$  powder, and then mixed with alginate, taken into syringe and wait for about 15 minutes, the formula is ready to be injected.

- In addition to test tube mixing on a vortex, experiments also were run  
10 which show that syringe mixing of this formula exhibited the same gelling characteristics. Experiments were also performed wherein chondrocyte cells were added into the formula, and it successfully gelled up at 14 minutes at room temperature, but remained injectable for more than two hours. Later experiments show that this formula can maintain its injectability for more  
15 than two weeks. Table 2 lists the components in this formula.

**Table 1. Components in Formula**

	Material	Working Conc.	Amount	Final Conc.
	alginate <sup>1</sup>	20 mg/mL	2.25 mL	15 mg/mL
	Cells in M199	120 million/mL	0.5 mL	20million/mL
20	$CaSO_4 \cdot 2H_2O$	powder	45 mg	15 mg/mL
	$CaCl_2 \cdot 2H_2O$ <sup>2</sup>	18 mg/mL	0.25 mL	1.5 mg/mL

<sup>1</sup> alginate used is UP MVG grade, dissolved in  $K_2HPO_4$  buffer

<sup>2</sup>  $CaCl_2$  is dissolved in M199 medium.

25

**EXAMPLE 3. Temperature Effect on Gelling Time**

- The time required for alginate to gel when mixed with calcium-chloride/calcium sulfate salt mixture varies with temperature. Sodium alginate, grade UP MVG from Pronova, was prepared as 2% solution in 0.1  
30 molar phosphate buffer, pH=7.4, containing 0.79% sodium chloride. 2.25 ml of alginate solution was mixed with 0.5 ml of M199 cell culture medium.

-38-

To this mixture, was added 0.25 ml of cell culture medium containing 18 mg calcium chloride per ml and 45 mg suspended calcium sulfate powder. After mixing, each sample was held at the indicated temperature and tested periodically for gel formation. The time at which gel formation was detected in duplicate samples is shown in the accompanying table. At room temperature (20-30°C) gel formed from 13 to 30 minutes. At 37°C, gel formation took 7-8 minutes.

**Table 2. Time to Gel at Different Temperatures**

	Temperature (C)	Gelling Time (min)
10	50	5, 6
	40	7, 7
	37	7, 8
	35	9, 9
	30	13, 14
15	25	17, 18
	20	28, 30
	15	53, 54
	10	120, 115
	5	240, 240
20	0	120 < t < 19 hr.

**EXAMPLE 4. Formation of Injectable Alginate Gel**

The sensitivity of the time for gel formation to slight variations in the concentration of various components in the mixture was tested as described below. Solutions of sodium alginate, calcium chloride, and calcium sulfate, were prepared as described as in Example 1 and 2, except that the amount of one or another component was varied by plus or minus 16% of the base value. The samples were held at room temperature after mixing, and tested periodically for gel formation.



-39-

The time for gel formation and the period during which the consistency remained suitable for injection are shown in Table 3. The amount of each component is indicated in the table as M for the base value, H for 16% above the base value, and L for 16% below the base value. Within the variations  
5 of these parameters, the gel time was 18 minutes plus or minus about 15%, indicating little effect of variation in any particular component within this range. Although all of these solutions gelled within about 20 minutes, the consistency of the alginate gel was still acceptable for injection beyond 24 hours later. Samples of this formulation prepared in a similar manner  
10 demonstrated suitable injectability properties over one month after formulation.

-40-

Table 3. Time for Gel Formation

No. of Expts.	Algin-ate	M199	CaSO <sub>4</sub>	CaCl <sub>2</sub>	Gel Time (min)	Inject-ability	Ambient Temp. (°C)
5	4	M	M	M	18,18,18,17	> 24 hr	23
	4	M	M	<u>H</u>	18,18,17,16	> 24 hr	23
	6	M	M	<u>L</u>	20, 20, 19, 20, 20, 19	> 24 hr	21
	2	H	H	H	18, 19	> 24 hr	22
	2	L	L	L	18, 19	> 24 hr	22
10	2	<u>H</u>	M	M	19, 19	> 24 hr	22
	2	<u>L</u>	M	M	18, 17	> 24 hr	21
	2	M	<u>H</u>	M	20, 21	> 24 hr	21.5
	2	M	<u>L</u>	M	19, 19	> 24 hr	21.5
	2	M	M	<u>H</u>	17, 18	> 24 hr	22
15	2	M	M	<u>L</u>	21, 20	> 24 hr	22
	H (+16%)	2.6 ml	0.58 ml	0.052 g	0.29 ml		
	M	2.25	0.5	0.045	0.25		
	L (-16%)	1.9	0.42	0.038	0.21		

Notes:

1. Alginate concentration is 2% by weight/volume

2. CaCl<sub>2</sub> concentration is 18mg/ml in M199 medium

**Example 5. Comparison of  $\text{CaSO}_4$  powder to  $\text{CaCl}_2/\text{CaSO}_4$  mixtures**

This experiment was performed to quantitatively characterize the gel and gelling process of two formulations and qualitatively determine the strength of the final gels. During the experiments described above, gelation and the time needed to set gel were determined by visual observation. By using a viscometer, the viscosity change when alginate starts to gel up can be directly measured, and also the relative strength of the gels can be qualitatively determined.

Alginate gelled by  $\text{CaSO}_4$  powder was prepared as described for Example 1, and alginate gelled by a mixture of  $\text{CaCl}_2/\text{CaSO}_4$  was prepared as described for Example 2. The  $\text{CaCl}_2/\text{CaSO}_4$  samples were prepared with syringe mixing, and  $\text{CaSO}_4$  powder samples were prepared with vortex mixing method. Syringe mixing allows a very simple procedure that is easy to perform and yields a very consistent product. It also permits the use of approved biomedical devices, and creates a closed system to segregate product from environmental exposure. Viscosity changes along the gelling course of the two systems at temperatures of  $5^\circ\text{C}$ ,  $25^\circ\text{C}$  and  $37^\circ\text{C}$  were measured and the result are plotted in Figure 1 through Figure 3.

At room temperature, the  $\text{CaCl}_2/\text{CaSO}_4$  sample gelled to an injectable set point<sup>1</sup> at 13 minutes, while the  $\text{CaSO}_4$ -only sample did not set the gel until minute 56. The viscosity of the  $\text{CaCl}_2/\text{CaSO}_4$  sample before gelling is about 665 cP, and  $\text{CaSO}_4$ -only sample is 202 cP; the former is approximately 3.3 times as thick as that of the latter. When the alginate starts gelling, its viscosity increases abruptly and reaches very high values (Fig. 1), going from 1100 cP to 30,000 cP in 2 minutes. After gelling, the viscosity of the  $\text{CaCl}_2/\text{CaSO}_4$  sample is about 2 times that of  $\text{CaSO}_4$ -only sample (about 40,000 cP vs. 20,000 CP).

Gelation of both formulations are accelerated at body temperature.  $\text{CaCl}_2/\text{CaSO}_4$  sample starts gelation at 4 minutes, and  $\text{CaSO}_4$ -only sample

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<sup>1</sup> Injectable set point: consistency of gel that can be stacked without collapsing.

-42-

gels up at 13 minutes. The viscosities before gelling are about 520 and 135 cP respectively for  $\text{CaCl}_2/\text{CaSO}_4$  sample and  $\text{CaSO}_4$ -only sample at  $37^\circ\text{C}$ . The lower initial viscosity is due to the higher temperature. After gelation, the viscosity of the  $\text{CaCl}_2/\text{CaSO}_4$  sample could reach 16,600 cP which is approximately 10 times higher than that of  $\text{CaSO}_4$ -only formula (Fig. 2), indicating that mixing  $\text{CaCl}_2$  and  $\text{CaSO}_4$  produces a stronger gel which may permit a better tissue separation in implantation application.

Gelation is dramatically retarded at lower temperatures. Similarly, the  $\text{CaCl}_2/\text{CaSO}_4$  sample is more viscous than the  $\text{CaSO}_4$ -only formula at all time points at  $5^\circ\text{C}$  (Fig. 3).

Compared to the  $\text{CaSO}_4$ -only formula, using a mixture of  $\text{CaCl}_2$  and  $\text{CaSO}_4$  gives: 1) 3 to 4 times higher initial viscosity; 2) much shorter time to reach injectable consistency; 3) higher viscosity after gelation, thus a better tissue separation.

**Example 6. Experiments conducted to demonstrate a composition of alginate gel having the characteristics of tissue compatibility and injectability.**

A composition was prepared containing the components listed in Table 4 below. This composition had viscosity of approximately 1000 Cp immediately post-mixing. Gelation occurred at approximately 15 minutes post-mixing and produced an intact, but weakly cross-linked gel (modulus= $6.59 \text{ kgf/cm}^2$ ). When injected through a 22-gauge needle, the gel was fractured into irregularly shaped pieces ("crumbles"), resulting in decrease in gel strength (modulus= $.231 \text{ kgf/cm}^2$ ).

This material was injected into subcutaneous sites in normal mice, and the implant monitored for six months. Upon histological examination of the implant, crumbles were observed of dimension ranging from 50 microns to 500 microns. The original volume of the implant persisted for six months. Histological examination over time showed loose connective tissue from the host enveloping the crumbles, indicating that injection of alginate gel having

-43-

the composition described herein into subcutaneous sites promotes an infiltration of loose connective tissue in and around the injected gel fragments. This gel exhibits the property of inciting a fibrotic response, and through this response, connective tissue ultimately occupies a significant proportion of the bulk created upon injection of the gel material.

**Table 4. Ingredients**

Ingredient	Volume	Final Concentration
alginate (PRONOVA UP-MVG)	2.25 mL	15 mg/mL
M199	0.50	
CaCl <sub>2</sub> ·2H <sub>2</sub> O in M199	0.25 mL	1.5 mg/mL
CaSO <sub>4</sub> ·2H <sub>2</sub> O		15 mg/mL

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, reconstructive surgery, cell culture, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

**CLAIMS:**

1. A composition for injection into an animal comprising a biocompatible polymer; said biocompatible polymer forming a stable, partially hardened hydrogel.
- 5 2. The composition of claim 1, wherein the composition is a paste which resists flow under a force equal to the force of gravity when formed into a block, said paste being suitable for injection into an animal.
3. The composition of claim 2, wherein the injectable paste consists essentially of a suspension of irregularly shaped hydrogel particles,  
10 said particles having diameter of from 30 microns to 500 microns.
4. The composition of claim 3, wherein the injectable paste has gel strength of at least 0.1 kgf/cm<sup>2</sup> and the hydrogel in the particles has a gel strength of at least 3 kgf/cm<sup>2</sup>.
5. The composition of claim 1, wherein the composition is  
15 prepared by forming a friable hydrogel and forcing the hydrogel through an orifice whereby the hydrogel is fractured forming irregularly shaped particles, the composition containing said particles retaining consistency sufficient to separate tissue planes in an animal.
6. The composition of any one of claims 1-5, wherein the  
20 biocompatible polymer is alginate.
7. The composition of claim 6, further comprising a highly soluble salt of a multivalent metal cation, a sparingly soluble salt of a multivalent cation.
8. The composition of claim 7, wherein the highly soluble salt and  
25 the sparingly soluble salt are both calcium salts.
9. The composition of claim 7, wherein the highly soluble salt is calcium chloride and the sparingly soluble salt comprises a cation selected from the group consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin, and di-, tri- or tetra-functional organic cations and an  
30 anion selected from the group consisting of low molecular weight dicarboxylic acid ions, sulfate and carbonate.

-45-

10. The composition of claim 9, wherein the sparingly soluble salt is calcium sulfate and the mixture contains calcium chloride/calcium sulfate in ratio of from 0.0075 to 1.0 by weight.

11. The composition of claim 9, wherein the mixture contains at  
5 least 0.5 % alginate by weight and at least about 0.001 g calcium chloride per gram of alginate.

12. The composition of claim 9, further comprising a sequestrant which competes with alginate for binding of calcium ion.

13. The composition of claim 12, wherein the sequestrant comprises  
10 phosphate anion.

14. The composition of any one of claims 1-13, wherein the composition also comprises living cells.

15. The composition of claim 14, wherein the cells are selected from the group consisting of chondrocytes and other cells that form cartilage,  
15 osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells.

16. The composition of any one of claims 14-15, wherein the cells are selected from the group  
consisting of dissociated cells and cell aggregates.

20 17. A method for treating anatomical defects in an animal in need of treatment thereof, the method comprising implanting into the animal at the site of the defect a composition according to any one of claims 1-16.

18. A method for treating anatomical defects in an animal in need of treatment thereof, the method comprising

25 preparing a biodegradable, biocompatible hydrogel which is partially hardened, said partially hardened hydrogel optionally containing living cells,

fracturing the hydrogel by passing the hydrogel through an orifice under pressure to produce a suspension containing hydrogel particles, and  
implanting the suspension into the animal at the site of the defect,

30 wherein the hydrogel suspension after implantation induces fibrotic response by surrounding tissue and vascularization of the implant.

-46-

19. A method for treating anatomical defects in an animal in need of treatment thereof, the method comprising

preparing a biocompatible hydrogel comprising a biopolymer or a modified biopolymer, optionally containing living cells,

5 passing the hydrogel through an orifice under pressure to produce a flowable composition having consistency sufficient to divide tissue planes, and

implanting the flowable composition into the animal at the site of the defect.

10 20. A kit for preparing a partially hardened hydrogel solution suitable for injection into an animal which hydrogel will fully harden *in vivo*, said kit comprising:

a polymer which forms a hydrogel upon cross-linking by divalent cations, said polymer preferably being alginate;

15 a fully soluble salt containing a divalent metal cation, said soluble salt preferably being calcium chloride; and

a sparingly soluble salt having a cation selected from the group consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin, and di-, tri- or tetra-functional organic cations; and an anion selected from  
20 the group consisting of low molecular weight dicarboxylic acids, sulfate ions and carbonate ions, said sparingly soluble salt preferably being calcium sulfate;

wherein the components are provided such that upon combination with a suspension of mammalian cells they will form within 30 minutes a partially  
25 hardened hydrogel suitable for injection, having the polymer at a concentration of 1.5%, the fully soluble salt at a concentration of 1.5 mg/L, and the sparingly soluble salt at a concentration of 15 mg/L, and further wherein said partially hardened hydrogel will not under go substantial change in viscosity for at least 4 hours.

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-47-

21. A kit for preparing a composition according to any one of claims 1-16 for injection into an animal comprising (a) a biocompatible polymer, said biocompatible polymer being capable of forming a hydrogel upon cross-linking with multivalent cations, (b) a highly soluble salt of a multivalent metal cation, and (c) a sparingly soluble salt of a multivalent cation.

22. A kit for preparing a composition according to any one of claims 6-11 for injection into an animal comprising (a) a biocompatible polymer, said biocompatible polymer being capable of forming a hydrogel upon cross-linking with multivalent cations, (b) a highly soluble salt of a multivalent metal cation, and (c) a sparingly soluble salt of a multivalent cation, wherein the biocompatible polymer and the sparingly soluble salt are in powder form, optionally in a single container.

23. The kit of claim 22, further containing a sequestrant which competes with alginate for binding of calcium ion.

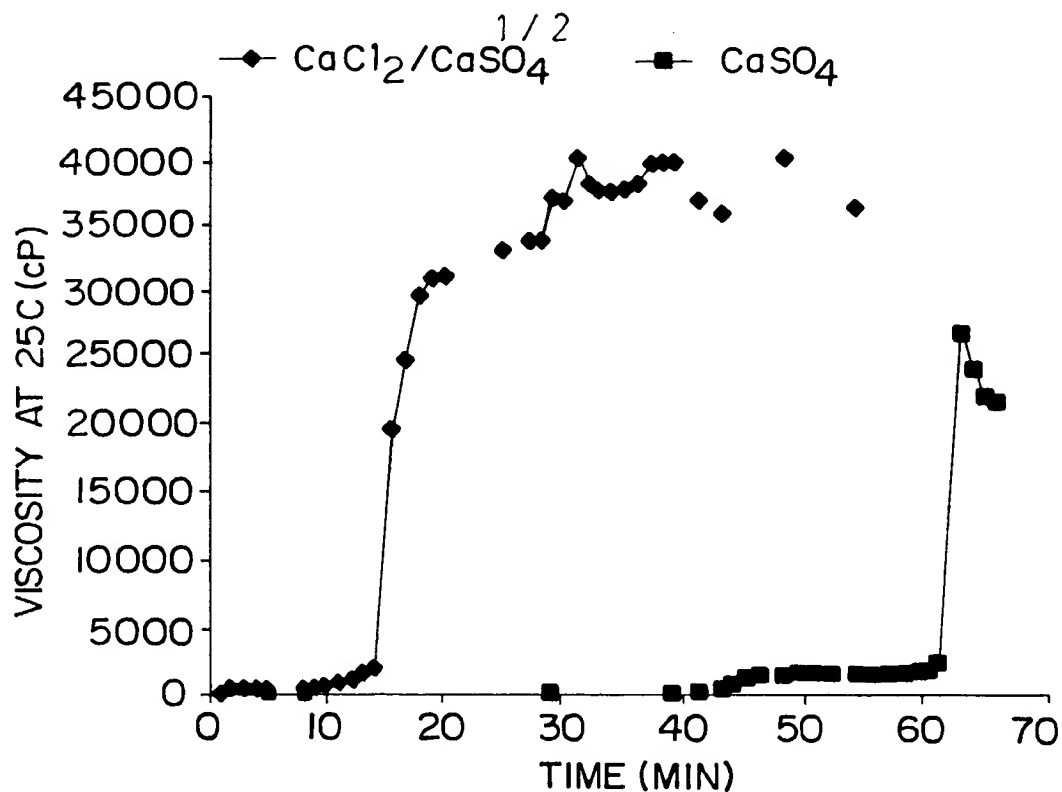


FIG. 1

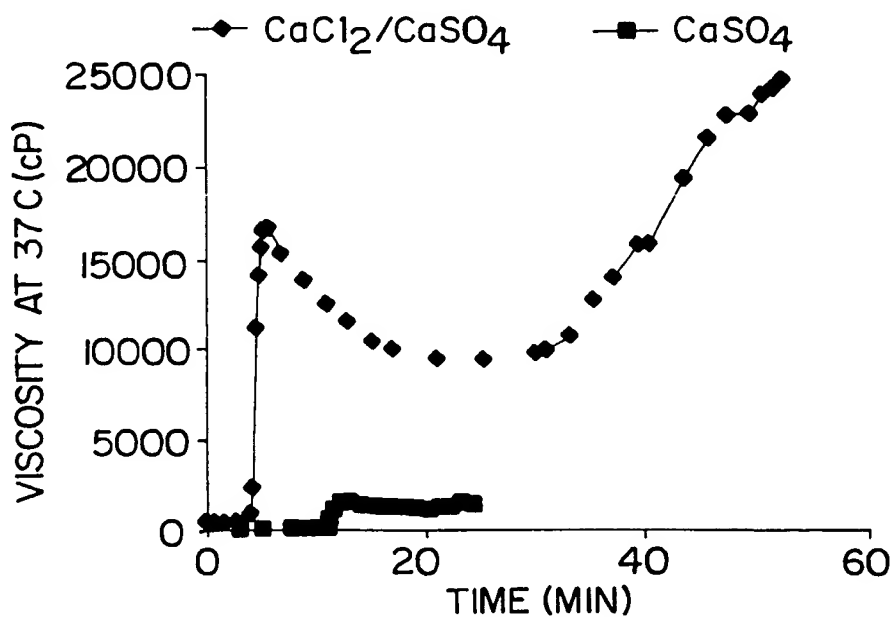


FIG. 2

2 / 2

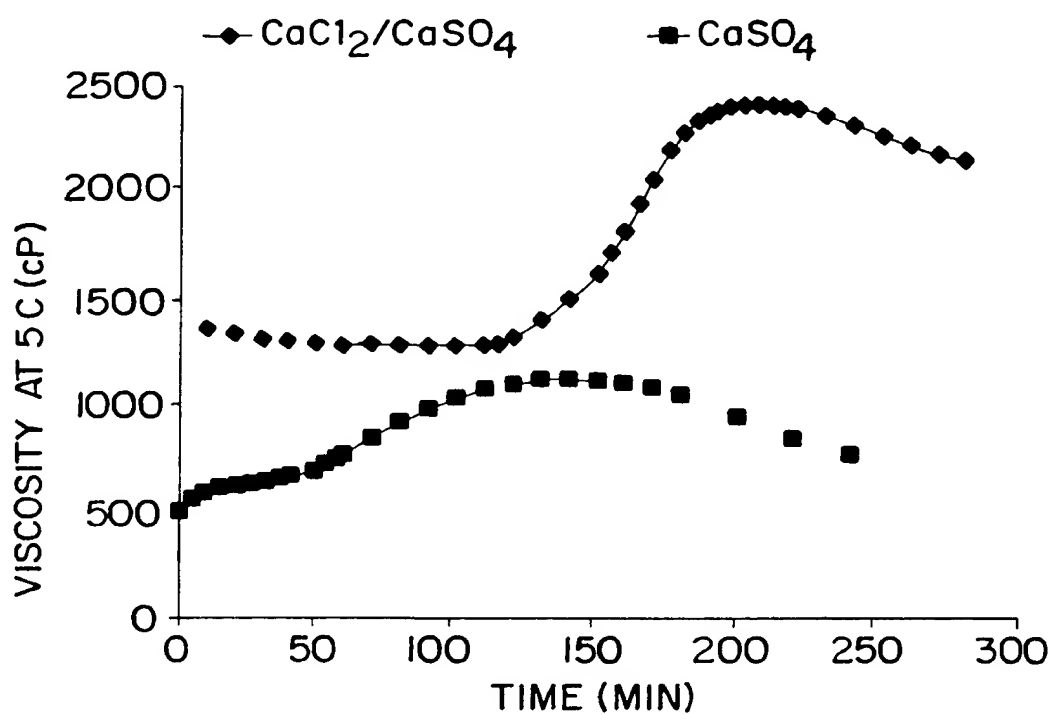


FIG. 3

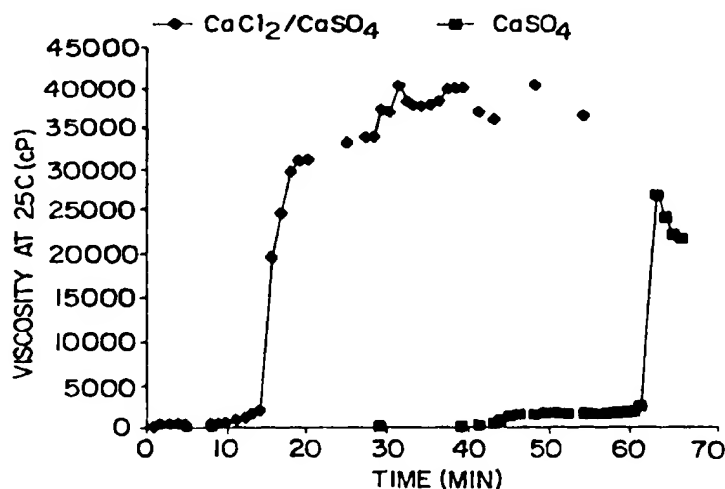




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## (57) Abstract

This invention provides compositions for use in implanting into an animal comprising a biodegradable, biocompatible polymer which forms a hydrogel upon cross-linking. In a preferred mode, the polymer is cross-linked by multivalent ions; more preferably, a soluble salt of a multivalent ion and a sparingly soluble salt of a multivalent ion, these components being combined into a mixture which forms a partially hardened, injectable hydrogel, the consistency of the mixture being suitable for implanting the partially hardened hydrogel mixture into the animal, where the implanted, partially hardened hydrogel forms *in situ* a fully hardened hydrogel. Preferably, the mixture also contains a biocompatible sequestrant which competes with the biocompatible polymer for binding of the multivalent cross-linking ion. The composition may or may not contain living cells. The invention also provides methods for implanting the composition in an animal, optionally as an implant containing living cells.

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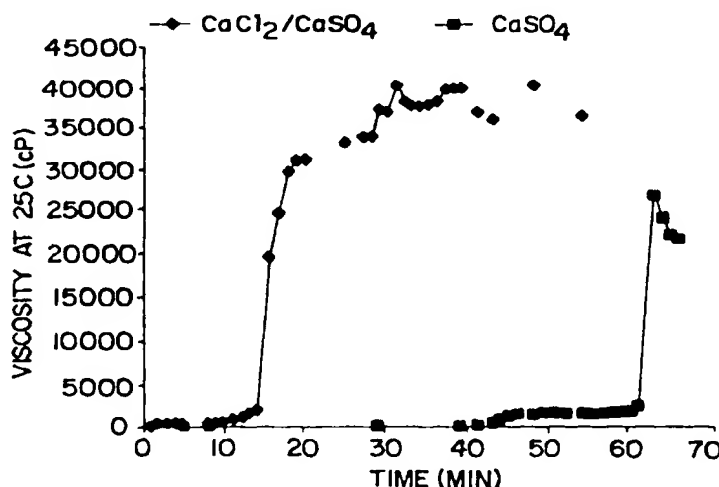
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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US Not furnished (CIP) Filed on Not furnished			
(71) Applicant (for all designated States except US): REPROGENESIS, INC. [US/US]; 21 Erie Street, Cambridge, MA 02139 (US).		Published With international search report.	
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(54) Title: IMPROVED HYDROGEL FOR TISSUE ENGINEERING



## (57) Abstract

This invention provides compositions for use in implanting into an animal comprising a biodegradable, biocompatible polymer which forms a hydrogel upon cross-linking. In a preferred mode, the polymer is cross-linked by multivalent ions; more preferably, a soluble salt of a multivalent ion and a sparingly soluble salt of a multivalent ion, these components being combined into a mixture which forms a partially hardened, injectable hydrogel, the consistency of the mixture being suitable for implanting the partially hardened hydrogel mixture into the animal, where the implanted, partially hardened hydrogel forms *in situ* a fully hardened hydrogel. Preferably, the mixture also contains a biocompatible sequestrant which competes with the biocompatible polymer for binding of the multivalent cross-linking ion. The composition may or may not contain living cells. The invention also provides methods for implanting the composition in an animal, optionally as an implant containing living cells.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/22859

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61L27/00 A61L31/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 25080 A (MASSACHUSETTS INST TECHNOLOGY ;CHILDRENS MEDICAL CENTER (US)) 10 November 1994 cited in the application see the whole document ---	1-23
P,X	WO 96 40304 A (REPROGENESIS INC) 19 December 1996 see page 16, line 17 - line 29; claims ---	1-7, 14-19
Y	DE 41 03 876 A (ASAHI OPTICAL CO LTD) 14 August 1991 see claims; examples ---	1-23
P,Y	WO 97 17038 A (UNIV MASSACHUSETTS) 15 May 1997 see page 10, line 18 - line 33; claims; examples ---	1-23
-/-		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

24 June 1998

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 459 733 A (PROTAN BIOPOLYMER AS) 4 December 1991 see page 3, line 19 - line 58; claims ---	1
A	EP 0 727 232 A (MENTOR CORP) 21 August 1996 -----	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 97/22859

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 17-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/22859

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